International Immunopharmacology 54 (2018) 169–176

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Toll-like receptor 4 deficiency increases resistance in sepsis-induced immune dysfunction

Chao Cao[a], Yanfen Chai[a], Songtao Shou[a], Jun Wang[a], Ying Huang[a], Tao Ma[b,*]

[a] Department of Emergency Medicine, Tianjin Medical University General Hospital, Tianjin, China
[b] Department of General Surgery, Tianjin Medical University General Hospital, Tianjin, China

ARTICLE INFO

Keywords:
Toll-like receptor 4
Sepsis
Immune dysfunction
Regulatory T cell
Inflammatory mediator

ABSTRACT

Sepsis constitutes a serious life-threatening syndrome associated with complications of deregulated inflammatory response against endotoxin/lipopolysaccharide (LPS)-mediated severe infection. Toll-like receptor 4 (TLR4) plays a critical role in the activation of innate immunity through recognition of LPS. However, the impact of TLR4 signaling on the development of sepsis-induced immune dysfunction remains unclear. The aim of this study was to investigate the effect of TLR4 on regulatory T cells (Tregs) and its potential mechanism. To simulate sepsis, male C57BL/6 (wild-type) and C57BL/10ScNJNJU (TLR4$^{-/-}$) mice were subjected to cecal ligation and puncture (CLP). After 24 h, pro- and anti-inflammatory cytokine secretion, neutrophil and macrophage lung and liver infiltration were assessed to evaluate the sepsis-induced inflammatory response. The quantity and apoptotic rate of Tregs were measured. The expression of cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and forkhead/winged helix transcription factor p3 (Foxp3) were analyzed. Cytokine (i.e., TNF-α, IL-2, IL-10, and IL-4) secretion by Tregs in the cell suspensions and the suppressive activity on CD4$^+$CD25$^-$ T cell proliferation were also determined in vitro. At 24 h after the CLP procedure, the wild-type mice exhibited increased Treg levels and expression, and secreted inflammatory factors in the serum were markedly overproduced. However, the TLR4$^{-/-}$ mice attenuated the increased Treg expression and inflammatory factor overproduction. These results indicate that in a model of post-septic mice, TLR4 deficiency improves immune paralysis by attenuating Treg activity and restoring a pro-inflammatory cytokine balance. Thus, modulation of the TLR4 activity may be useful in preventing immune dysfunction in sepsis.

1. Introduction

Sepsis is denoted as a complex infectious syndrome that leads to severe sepsis, endotoxin shock, organ dysfunction, or eventual death. The complex immune response associated with sepsis results in a high rate of morbidity and mortality, despite more differentiated treatment options and clinical advances [1–3]. Increasing evidence suggests that innate immune and inflammatory responses are involved in the pathophysiology of sepsis, whereby the bacterial components, especially lipopolysaccharide (LPS), activate an inflammatory response that is accompanied with the release of inflammatory mediators.

Pattern recognition receptors (PRRs) are essential for activation of the innate immune response. Among PRRs, Toll-like receptors (TLRs) comprise a family of pathogen-associated PRRs for the detection of microbial components, which play a fundamental role in the pathogen recognition of innate immune and inflammatory responses [4,5]. In particular, TLR4, as an essential receptor for LPS signaling, may trigger the activation of an extracellular signaling pathway and result in the excessive release of inflammatory mediators [6]. Previous studies have demonstrated that the expression of TLR4 was elevated on human monocytes in healthy volunteers challenged with LPS [5,7], as well as in patients with sepsis [8,9].

Numerous studies have demonstrated that naturally occurring CD4$^+$CD25$^+$ regulatory T cells (Tregs) play a pivotal role in the persistent immunosuppressive and immune paralysis state, which might be associated with the increasing morbidity and mortality in patients with sepsis. Tregs are defined by expression of the forkhead/winged helix transcription factor p3 (Foxp3) transcription factor, the absence of which causes a fatal T cell-mediated lymphoproliferative and autoimmune disorder both in animals and humans [10,11]. Notably, it has been previously demonstrated that sepsis leads to a relative increase in the number of Tregs and their suppressive function both in clinical [12,13] and experimental studies [14,15], which establishes these cells as an important participant in the inhibition of immune responsiveness during sepsis.

In our previous study we have demonstrated that TLR4 is over-

* Corresponding author at: Department of General Surgery, Tianjin Medical University General Hospital, Tianjin 300052, China.
E-mail address: caohuachao@sina.com (T. Ma).

https://doi.org/10.1016/j.intimp.2017.11.006
Received 3 September 2017; Received in revised form 4 October 2017; Accepted 5 November 2017

expressed on the surface of Tregs during sepsis, suggesting its involvement in affecting the immune microenvironment and immunopathology processes, potentially by impacting certain signaling pathways or cytokine network. However, the molecular mechanisms that contribute to Treg depletion and the development of functional abnormalities remain to be elucidated, and the specific contribution of TLR4 signaling to the development of sepsis-induced immune dysfunction is poorly understood. We hypothesized that variations in TLR-dependent signaling might modulate the maturation process and/or survival of Tregs after polymicrobial sepsis. To test this hypothesis, we assessed the early (24 h) quantitative and functional features of spleen Tregs in wild-type and TLR4 deficient mice ($TLR4^{-/-}$) in a model of polymicrobial sepsis.

2. Materials and methods

2.1. Animal handling and care

Male C57BL/6 (wild-type, WT) mice and age matched C57BL/10ScNJNJU ($TLR4^{-/-}$) mice used in our experiments (weight range 18–22 g) were provided by the Model Animal Research Center of Nanjing University, Nanjing, China. All animals were housed in separate cages in a temperature-controlled room with 12 h light and 12 h darkness to acclimatize for at least 7 days before being sacrificed. All animals had free access to water but were fasted overnight prior to the experiment. All experimental manipulations were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Burns Institute, the First Affiliated Hospital of PLA General Hospital, Beijing, China.

2.2. Sepsis model and survival rate

We used an experimental model of polymicrobial sepsis induced by cecal ligation and puncture (CLP). CLP was performed to induce sepsis in mice as previously described [16]. Briefly, the mice were anesthetized by 5.0% isoflurane. A midline incision was made on the anterior abdomen and the cecum was exposed and ligated. Then, two punctures were made through the cecum with a needle and feces were extruded from the holes. The abdomen was then closed in two layers. Sham surgically operated mice served as the surgery control group. Immediately after surgery, a single dose of resuscitative fluid (lactated Ringer's solution, 50 mL/kg body weight) was administered by subcutaneous injection. The survival experiments were performed in three groups of mice: Sham, WT CLP, and $TLR4^{-/-}$ CLP. In addition, an observational experiment for survival rate over 72 h was carried out ($n = 10$/group).

2.3. Accumulation of neutrophils and macrophages in lung and liver tissues

Neutrophil and macrophage accumulation in the lung and liver tissues was examined by staining with hematoxylin and eosin solution (Sigma Aldrich, St. Louis, MO, USA) to estimate inflammation. From each block, three sides were evaluated, counterstained with hematoxylin, and examined using bright field microscopy. The results are expressed as the numbers of neutrophils and macrophages/field (200 ×).

2.4. Enzyme-linked immunosorbent assay (ELISA)

After 24 h following model establishment, the release of pro-inflammatory cytokines, TNF-α and IL-2, as well as anti-inflammatory cytokines, IL-10 and IL-4, was measured using commercially available enzyme-linked immunosorbent assay kits (Bioscience, San Diego, CA, USA) according to the instructions provided by the manufacturer.

2.5. Isolation of splenic $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^-$ T cells

Mononuclear cells were obtained from collagenase D-treated spleen preparations of mice following Ficoll-Paque density gradient centrifugation. Splenic cells were dissociated through a 30-mm stainless steel mesh and treated with erythrocytolysin. $CD4^+$ $CD25^+$ Tregs were isolated from the mononuclear cells using a mouse $CD4^+$ $CD25^+$ regulatory T cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) and a MiniMACS™ separator with a positive selection MS/LD column according to the manufacturer's instructions. The viability of $CD4^+$ $CD25^+$ Tregs was approximately 98% as determined directly after the purification process by means of trypan blue exclusion, and the purity of $CD4^+$ $CD25^+$ Tregs was 94.94 ± 3.20% as assessed by flow cytometry. $CD4^+$ $CD25^+$ Tregs were resuspended and cultured at 37 °C in 5% CO_2 in humidified air overnight for recovery. The percentage and number of Tregs in $CD4^+$ T lymphocytes were detected.

2.6. Quantity and apoptotic rate of Tregs

To assess the apoptotic rate of Tregs in the CLP models, Tregs were isolated using immunomagnetic beads, which were stained with Annexin-V-FITC (Bioscience) and propidium iodide (Bioscience). The apoptotic rate of Tregs was determined by flow cytometry after 1 h.

2.7. SYBR green real-time reverse transcription-polymerase chain reaction (RT-PCR)

Approximately 2×10^5 cells/sample were prepared for extracting total RNA using the single-step technique of acid guanidinium thiocyanate-chloroform extraction according to the manufacturer's instruction (Eppendorf, Hamburg, Germany). The concentration of the purified total RNA was spectrophotometrically determined at 260 nm. mRNA expression of Foxp3 and TLR4 in cells was quantified by SYBR Green 2-step, real-time RT-PCR. After removal of potentially contaminating DNA with DNase I, 1 μg of total RNA from each sample was used for RT with oligo dT and Superscript II to generate first-strand cDNA. The PCR mixture was prepared using SYBR Green PCR Master Mix obtained from Invitrogen (Invitrogen, Shanghai, China). The primer sequences were as follows: *Foxp3*, forward: 5′-CAG CTG CCT ACA GTG CCC CTA G-3′, and reverse: 5′-CAT TTG CCA GCA GTG GGT AG-3′; *Tlr4*, forward: 5′-TTT CAC CTC TGC CTT CAC TAC A-3′; and reverse: 5′-AGA TAC ACC AAC GGC TCT GAA T-3′. The amplification PCR consisted of 1 min denaturation step at 95 °C followed by 40 cycles of 15 s at 95 °C and 40 s at 60 °C on a Sequence Detection System (Eppendorf, Hamburg, Germany). All samples were run in quadruplicate.

2.8. Flow cytometry

To observe the expression of cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) on the surface of Tregs, cells were stained with mouse anti-mouse CTLA-4-FITC antibody (Bioscience) for 30 min at 4 °C in the dark. The same procedure was implemented for TLR4 detection. Concomitantly, for detection of intranuclear Foxp3, cells were reacted with 1 mL freshly prepared fixation/permeabilization working solution for 2 h at 4 °C. After washing cells with 1 × permeabilization buffer twice, cells were stained with anti-mouse/rat Foxp3-FITC antibody (Bioscience) for 30 min at 4 °C in the dark. After being washed twice, cells were analyzed by flow cytometry using a FACScan (BD Biosciences, San Jose, CA, USA). The fluorescence strength was represented by the value of the mean (mean fluorescence intensity).

2.9. Cell culture and stimulation

Cell cultures were incubated at 37 °C (5% CO_2) in RPMI 1640 medium (Gibco, Eggenstein, Germany), supplemented with 10% fetal